Biochem. J. (1969) **111**, 487
Printed in Great Britain

A Phosphorylation Site in Brain and the Delayed Neurotoxic Effect of some Organophosphorus Compounds

By M. K. JOHNSON
Biochemical Mechanisms Group, Toxicology Research Unit, Medical Research Council Laboratories, Carshalton, Surrey

(*Received* 6 *August* 1968)

1. It is proposed that part of a neurotoxic dose of di-isopropyl phosphorofluoridate will be covalently bound *in vivo* to a specific component in the brain and spinal cord as the initial biochemical event in the genesis of the lesion. 2. A test system *in vitro* was devised that removes many di-isopropyl phosphorofluoridate-binding sites and indicates that the specific component may be a protein present in brain at a concentration comparable with that of the cholinesterases. 3. The site was found to be present and capable of binding di-isopropyl phosphorofluoridate *in vitro* in brain samples taken from either normal hens or those dosed with organophosphorus esterase inhibitors that are not neurotoxic. 4. Very little of the specific binding activity was found in brain samples from hens pre-dosed with a variety of neurotoxic organophosphorus compounds. 5. A solubilized preparation of the active brain component was obtained, suitable for further purification and study.

The inhibition of acetylcholinesterase and the consequent acute cholinergic effect of DFP* and many other organophosphorus compounds *in vivo* is well known. There is, however, another, different, effect of DFP and some other organophosphorus compounds that is less well known, the mechanism of which is not understood. Symptoms are not seen until 10–15 days after a single dose of the agent. In adult hens an irreversible ataxia of the legs is produced and this is not modified by administration of atropine, eserine or cholinesterase reactivators before or after the neurotoxic agent; the lesions in nervous tissue have been described in detail by Cavanagh (1964). It has not so far been possible to find a relationship between chemical structure and activity in those compounds, and the whole problem of their neurotoxicity has been reviewed by Aldridge & Barnes (1967).

Low concentrations of toxic organophosphorus compounds react only slowly under physiological conditions with most chemical groupings found in tissue components. However, many of them react rapidly at the active site of some esterases to produce an inactive phosphorylated enzyme (Aldridge, 1956). It is therefore proposed that an essential early step in the neurotoxic process is an irreversible inhibition of an enzyme in nervous tissue. Moreover, if a neurotoxic dose of [^{32}P]DFP is injected into a hen, the relevant enzyme will be both inhibited and labelled. In an attempt to distinguish such an esterase from numerous others in the brain a method was developed that decreases the number of sites available for radioactive labelling. A quantity of protein sites that are probably connected with neurotoxicity was identified and obtained in soluble form. Preliminary accounts of this work have been given (Johnson, 1967; Aldridge, Barnes & Johnson, 1969).

*Abbreviations: DFP, di-isopropyl phosphorofluoridate; TEPP, tetraethyl pyrophosphate.

MATERIALS AND METHODS

Chemicals. Diethyl 4-nitrophenyl phosphate (Paraoxon), TEPP, 2 - hydroxyiminomethyl - *N* - methylpyridinium methanesulphonate (P.2.S) and *NN'*-di-*n*-butylphosphorodiamidic fluoride (Butafox) were obtained from the Chemical Defence Experimental Establishment, Porton Down, Salisbury, Wilts.; *NN'*-di-isopropylphosphorodiamidic fluoride (Mipafox) and tetraisopropyl pyrophosphoramide (Iso Ompa) were from Fisons Pest Control Co. Ltd., Saffron Walden, Essex; DFP was from Boots Pure Drug Co. Ltd., Nottingham; di-2-chloroethyl 4-nitrophenyl phosphate (PE 304) was from Cooper Technical Bureau, Berkhamsted, Herts.; tri-2-methylphenyl phosphate, tri-2-ethylphenyl phosphate and 2-methylphenyl diphenyl phosphate were from Coalite Chemical Co. Ltd., Chesterfield, Derbyshire; 4-bromo-3,6-dichlorophenyl isopropyl methylphosphonothionate (OMS 989) and 4-bromo-3,6-dichlorophenyl methyl ethylphosphonothionate (OMS 988) were from the World Health Organisation, Geneva, Switzerland; tris, EDTA and Brij 35 detergent (polyethylene glycol lauryl ether) were from British Drug Houses Ltd., Poole, Dorset; eserine sulphate was from Burroughs Wellcome and Co., London,

N.W. 1; Cab-O-Sil was from Packard Instrument Co., La Grange, Ill., U.S.A.; 2,5-diphenyloxazole was from Thorn Electronics Co. Ltd., Tolworth, Surrey; sodium deoxycholate was from E. Merck A.-G., Darmstadt, Germany. [^{32}P]DFP was supplied by The Radiochemical Centre, Amersham, Bucks., at a concentration of about 3mM in propylene glycol and specific radioactivity 40–80 μc/μmole; it was diluted in tris buffer just before use *in vitro*.

Animals and dosing. Hens (5–7 months old) of Rhode Island Red × Light Sussex strain were obtained from a local breeder and were fed on a standard layer's mash. At the time of testing their body weights were in the range 1·8–2·8kg. with most being 2·2–2·5kg. When the compound to be tested was known to be acutely toxic by an anticholinesterase action, atropine (20mg. subcutaneously) and oxime reactivator 2-hydroxyiminomethyl-*N*-methylpyridinium methanesulphonate (100mg./kg. intraperitoneally) were administered in 0·9% NaCl 10–15min. before the dose. The route of administration of each compound is indicated in Table 1, the choice depending on the quantity involved and its solubility in water. All the compounds used had been examined previously for neurotoxicity by Dr J. M. Barnes: the maximum tolerated dose of non-neurotoxic compounds and a minimum dose of neurotoxic compounds giving strong response in all birds tested was normally used. In all cases where birds were killed at short times after dosing, an equal number of similarly dosed controls were observed for 14–28 days for signs of ataxia. The dose of 1·7mg. of DFP/kg. is considerably higher than that reported to be necessary by Davies, Holland & Rumens (1960), who used much heavier and older birds, but 1mg./kg. was found to be only marginally effective in several of the lighter birds.

Tissues. Blood samples were obtained from a wing vein by using a plastic syringe without anticoagulant. Blood was clotted at 37° for 60min. in a polyethylene centrifuge tube and a serum sample was obtained as the supernatant after centrifuging at 1500*g* for 20min. Hens were killed by dislocation of the neck, and the brain was rapidly dissected out and chilled in cold buffer (see below); spinal cord was obtained similarly. Ox brain was obtained fresh from the slaughterhouse, cleaned of blood clots, roughly separated into pieces of cortex and white matter and frozen in liquid N_2 for transport to the Laboratory, where it was stored at −20° for 14 days. Portions were thawed out rapidly at 10° and homogenized immediately. Homogenates (10%, w/v) of brain or cord were prepared in buffer by using the rotating (1100rev./min.) smooth Perspex pestle as described by Aldridge, Emery & Street (1960) with 0·01in. difference in diameter of the tube and the pestle. The buffer used in all experiments was 10mM-tris–HCl, pH 8·0 at 20°, containing EDTA (0·2mM).

Extracts of brain. An unwashed particulate fraction and a soluble fraction were prepared from a homogenate prepared in 0·3M-sucrose in the tris–EDTA buffer by centrifuging at 105000$g_{av.}$ for 60min. in a Spinco model L ultracentrifuge; the particles were resuspended in buffer to the original homogenate volume.

A soluble extract containing 83–85% of the protein of total homogenate was prepared by incubating four 5ml. portions of homogenate (14%, w/v, in buffer) each with 5ml. of sodium deoxycholate (1·6%, w/v, in buffer) at 0° for 30–40min.; the insoluble glutinous residue was sedimented by centrifuging at 105000*g* for 60min. A 15ml. portion of soluble supernatant was passed through a column (23cm. × 3cm.) of Sephadex G-25 loaded with Brij 35 non-ionic detergent (0·03% in tris–EDTA buffer, pH 8·0 at 20°); the soluble protein was completely eluted as a slightly opalescent solution in 25ml.

Extracts were also prepared from freeze-dried specimens of whole hen brain by ultrasonic treatment for 10min. with the large probe of the MSE 60w instrument at 0–8° of a smooth ground suspension of the powder (400mg. dry wt. ≡ 2·4g. of original brain) in 15ml. of tris–EDTA buffer containing Brij 35 (0·03%); most insoluble material was then sedimented by centrifuging at 40000*g* for 20min.

Labelling experiments in vitro. In experiments with unlabelled inhibitors, 3ml. of homogenate was preincubated for 30min. at 25° in stoppered tubes with 1ml. of buffer alone or of buffer containing inhibitor. In the standard test, the concentration of TEPP was 16μM and that of Mipafox 128μM. [^{32}P]DFP in buffer (1ml.) at 25° was then added quickly and incubation was stopped after a further 6min. by rapid addition of 13ml. of ice-cold acetone–ether (2:1, v/v) or 0·12M-sulphosalicyclic acid. The mixture was cooled in ice for 10min. and the precipitate sedimented by centrifuging at 1000*g* for 10min. at 2°. The supernatant was discarded and the pellet was washed three times by dispersing it in 15ml. of wash fluid and resedimenting. For acid-precipitated samples the wash fluid was 0·15M-$HClO_4$ and for solvent-precipitated samples it was ether–ethanol–water (10:7:3, by vol.). When whole homogenates were investigated the organic solvents were used to denature and wash; when detergents were present the acid system was found preferable, giving tighter-packed pellets and no loss of protein on washing. Radioactivity was determined in the washed samples in suspension in 3% (w/v) Cab-O-Sil in toluene–Cellosolve (1:1, v/v) as described by Bruno & Christian (1961). The primary scintillator was 2,5-diphenyloxazole and addition of a secondary component was found to offer no improvement when samples were counted in a Packard model 4312 liquid-scintillation spectrometer at 2·7% gain with the discriminators set at 50–1000. Counting efficiency for a soluble standard was 96% under these conditions and was not affected by the presence of water on either of the chosen wash fluids (0·5ml.) or of unlabelled brain precipitates. These checks do not completely prove that radioactivity of suspended particles is being measured at the same efficiency. However, specimens of labelled homogenate that had been precipitated and washed with organic solvents were readily soluble in 10ml. of 98% (w/w) formic acid and the solution became perfectly clear and almost colourless on addition of 2ml. of butyl acetate. Radioactivity of these samples could be measured without addition of scintillators when the instrument was set to 50% gain with the discriminators set at 50–1000. Efficiency was 26% and was unaffected by small variations in the composition of the medium. The ratios of counts/min. of a standard labelled brain precipitate sample to those of an aqueous standard were the same under these conditions as when they were counted in the Cab-O-Sil suspension system. This shows that there was no self-quenching of the counts in Cab-O-Sil suspension.

Two tests of the washing procedure are described below.

(i) The most sensitive enzymes in a homogenate were partially inhibited by a very brief incubation (5–10sec.) under the usual conditions with a low concentration of

unlabelled DFP (0·22 μM). A 100-fold excess of [^{32}P]DFP (22 μM) was then added, rapidly mixed and the whole was denatured with acetone at $-5°$. In this experiment conditions would have permitted adsorption of label to particulate matter before denaturing but the label found bound after the usual three washes was only 320–340 counts/min. in four experiments. In a parallel experiment in which incubation was continued for 30 min. before denaturing, 20000 counts/min. were bound. Comparison of these results shows that the proportion of radioactivity bound by non-specific adsorption could be only a very small proportion of the whole.

(ii) Quadruplicate labelled specimens washed with organic solvents were compared after two had been further washed by dissolving in 98% (w/w) formic acid (1 ml.), diluting to 10 ml. with water and reprecipitating at 0° by addition of 2 ml. of 10 M-$HClO_4$. No significant decrease in labelling was found, although the chance of occlusion of soluble radioactivity must have been greatly decreased by this step.

RESULTS AND DISCUSSION

Development of a standard test for determination of phosphorylation sites relevant to neurotoxicity

Principle of the test. When brain homogenate is incubated *in vitro* with DFP many brain proteins (probably esterases) are phosphorylated. Included among these proteins are several esterases that have been shown not to be implicated in the genesis of neurotoxicity (Davison, 1953; Aldridge & Barnes, 1961, 1966). Theoretically it should be possible to remove some or all of these by preincubating the tissue with non-neurotoxic inhibitors: the enzyme X (that one involved in neurotoxicity) should survive to be labelled by [^{32}P]DFP added afterwards. If a neurotoxic inhibitor were added to the non-neurotoxic one in the preincubation then more enzymes including enzyme X would be inhibited and would not be labelled when [^{32}P]DFP was added. The difference in labelling after these two preincubations would represent one or more proteins including enzyme X and can be quantitatively assessed.

Choice of concentration of [^{32}P]*DFP.* Hens were dosed with sufficient [^{32}P]DFP to ensure a profound neurotoxic response (1·7 mg./kg.). After 4 hr. brains were removed and homogenized in buffer. Samples were precipitated and washed with organic solvents as described in the Materials and Methods section. Radioactivity equivalent to 630, 650, 720 and 910 $\mu\mu$moles of P/g. wet wt. of brain (mean 730) was found. The concentration of DFP required *in vitro* to produce a similar amount of labelling was then determined (Fig. 1). A concentration of 6·4 μM-DFP gave 500–700 $\mu\mu$moles of P bound/g. of brain with 30 min. incubation as described. In the standard test as finally established, tissue was incubated for only 6 min. with 26–32 μM-DFP after a 30 min. preincubation; mean labelling of normal

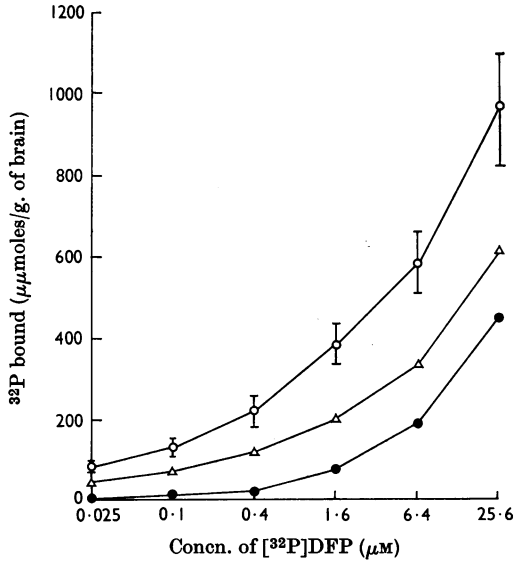

Fig. 1. Phosphorylation of hen brain incubated *in vitro* with [^{32}P]DFP. ○, Normal hen (mean ± S.D. of nine values); ● and △, 2 hr. and 8 days respectively after dosing with non-radioactive DFP. Conditions were as described in the Materials and Methods section, with 30 min. incubation with [^{32}P]DFP and no preincubation.

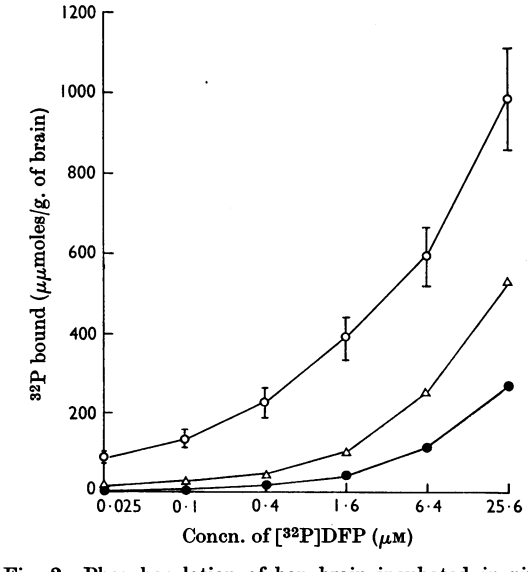

Fig. 2. Phosphorylation of hen brain incubated *in vitro* with [^{32}P]DFP. ○, Normal hen (mean ± S.D. of nine values); △, 2 hr. after subcutaneous administration of Mipafox (25 mg./kg.); ●, 2 hr. after subcutaneous administration of TEPP (10 mg./kg.). Conditions were as described in the Materials and Methods section, with 30 min. incubation with DFP and no preincubation.

hen brain under these conditions was 668 $\mu\mu$moles/g. (Table 1).

Choice of preincubation inhibitors. After initial trials TEPP and Mipafox were chosen as suitable inhibitors. Both are direct inhibitors of esterases *in vitro* (Aldridge, 1954) and the former is not neurotoxic whereas the latter is (Davies *et al.* 1960; Barnes & Denz, 1953). It is important to show that the failure of TEPP to produce a neurotoxic response is not due to failure to enter the brain when it is injected into hens. Fig. 2 shows that the number of phosphorylation sites in brain labelled by [^{32}P]DFP was drastically decreased after doses of either TEPP or Mipafox. Concentrations of these inhibitors *in vitro* were chosen so that the product of concentration and incubation time ($c \times t$) bore a relationship to $c \times t$ for DFP comparable with the relative molar doses of these compounds used *in vivo*. This choice ignores possible differences in distribution and breakdown of the compounds *in vivo*, but experiments are reported below that show that considerable variation in these concentrations do not invalidate the essential conclusions of the standard test.

Results of the standard test

In this test phosphorylation of hen brain homogenates by [^{32}P]DFP was determined after preincubation with (A) buffer only, (B) TEPP (16 μM)

Table 1. ^{32}P *bound* in vitro *to homogenates of brain of normal or dosed hens: effects of preincubations under the conditions of the standard test*

Hens (1·8–2·8 kg., paired for weight) were dosed as indicated in pairs, one with a neurotoxic compound and one with a structurally analogous non-neurotoxic compound, and killed after 4 hr. unless otherwise stated. Control dosed birds of similar weight were observed for at least 3 weeks for signs of neurotoxic effect. Labelling by 6 min. incubation with [^{32}P]DFP (32 μM) after preincubation for 30 min. with (A) buffer, (B) TEPP (16 μM) or (C) TEPP (16 μM) plus Mipafox (128 μM) was measured as described in the Materials and Methods section. Values were means of duplicates agreeing within 3%. Route of administration: p.o., oral; s.c., subcutaneous; i.p., intraperitoneal.

Compound	Dose (mg./kg.) and route of administration	^{32}P bound to brain ($\mu\mu$moles/g. wet wt.) after preincubation with: (A) Buffer only	(B) TEPP	(C) TEPP + Mipafox	Standard test difference (B) − (C) ($\mu\mu$moles/g.)
None (mean ± S.D. for eight undosed hens)		668 ± 149	215 ± 103	178 ± 102	37 ± 8‡
Non-neurotoxic					
Diethyl 4-nitrophenyl phosphate (Paraoxon)	15 s.c.	337	206	183†	23
		—	125	91†	34
Tetraisopropyl pyrophosphoramide (Iso Ompa)	250 p.o.	514	212	169	43
TEPP	10 s.c.	148	47	18	29
Tri-2-ethylphenyl phosphate*	1200 p.o.	438	107	86	21
		475	135	112	23
4-Bromo-3,6-dichlorophenyl methyl ethylphosphonothionate (OMS 988)	100 i.p.	234	90	76	14
		270	106	79	27*
Neurotoxic					
Di-2-chloroethyl 4-nitrophenyl phosphate (PE 304)	100 p.o.	348	182	172	10
NN'-Di-isopropylphosphorodiamidic fluoride (Mipafox)	25 s.c.	405	171	167	4
	30 s.c.	—	115	112	3
NN'-Di-*n*-butylphosphorodiamidic fluoride (Butafox)	0·3 s.c.	477	128	125	3
DFP	1·7 s.c.	146	75	72	3
		128	67	64	3
Tri-2-methylphenyl phosphate*	600 p.o.	569	193†	190	3
2-Methylphenyl diphenyl phosphate*	120 p.o.	317	88	83	5
		—	91	89†	2
4-Bromo-3,6-dichlorophenyl isopropyl methylphosphonothionate (OMS 989)	100 i.p.	211	86	80	6
		246†	84	80	4*

*Test performed 24 hr. after dosing.
†Single observation only.
‡About 1200 counts/min. for fresh [^{32}P]DFP.

or (C) a mixture of TEPP (16 μM) and Mipafox (128 μM). Table 1 shows the following. (*a*) Labelling by [^{32}P]DFP *in vitro* after preincubation with buffer (column A) is lower in homogenates from birds dosed with most of the compounds tested than in those from undosed birds; this shows that these compounds or metabolites of them enter the brain. (*b*) In homogenates of normal brain, phosphorylation sites exist that are removed by preincubation with Mipafox and TEPP (column C) but not by TEPP alone (column B); (B) – (C) is a measure of these sites. (*c*) After dosing with neurotoxic compounds having a wide range of structures and physical properties, the difference (B) – (C) is very much decreased; it is much less affected by doses of non-neurotoxic analogues. This result is strong evidence for the existence of a phosphorylation site in hen brain that is sensitive *in vitro* to DFP and Mipafox but not to TEPP at concentrations comparable with those used *in vivo*; the site is also sensitive *in vivo* to neurotoxic compounds at a minimum effective dose but much less sensitive to non-neurotoxic analogues at the highest practicable dose. Only one hen was used in each test so that some natural variation in the value of (B) – (C) is to be expected, but the division into two groups is clear and has not before been achieved on the basis of any other physical, chemical or biochemical characteristic.

Re-examination of tri-2-ethylphenyl phosphate and 4-bromo-3,6-dichlorophenyl methyl ethylphosphonothionate (Table 1). Table 1 shows that the difference (B) – (C) was smaller after dosing with these two compounds than with the other non-neurotoxic compounds. One dose of tri-2-ethylphenyl phosphate did not produce ataxia nor did several doses repeated at weekly intervals (Table 1 and Aldridge & Barnes, 1961; Bondy, Field, Worden & Hughes, 1960). However, 24hr. after the last of four oral doses of this compound (1200mg./kg.) spaced at 2-day intervals the value of (B) – (C) was 5 $\mu\mu$moles/g. and two out of two birds became ataxic; the correlation of neurotoxicity with depression of (B) – (C) was therefore maintained. Likewise the marginal effect of the single dose of 4-bromo-3,6-dichlorophenyl methyl ethylphosphonothionate can also be understood since two doses of this compound 3 days apart were found to produce typical ataxia in two out of four birds.

Effect of a non-neurotoxic dose of DFP. When a dose of DFP insufficient to cause ataxia in control hens (1mg./kg. in hens weighing only 1·8kg.), the value of (B) – (C) in the killed hen was not depressed (38 $\mu\mu$moles/g.). This result further strengthens the correlation as above.

Phosphorylation in vitro at various times after dosing of hens with DFP. At 8 days after dosing with DFP the quantity of all phosphorylation sites detected by a range of [^{32}P]DFP concentrations is markedly restored from the low values found 2hr. after dosing (Fig. 1). The difference (B) – (C) was also measured on the first day after dosing with DFP, and on days 14 (when ataxia was first seen), 17 and 28: the values were 6, 20, 20 and 13 $\mu\mu$moles/g. respectively. It may be concluded that a substantial initial decrease in the value of (B) – (C) is necessary for production of a neurotoxic

Table 2. *Effects of a range of concentrations of TEPP and Mipafox during preincubation on subsequent phosphorylation of hen brain homogenates by* [^{32}P]*DFP*

Experimental conditions were as described for the standard test in the Materials and Methods section. The two experiments, one with a fixed concentration of TEPP and one with a fixed concentration of Mipafox, each used the brain of a normal hen. (B) – (C) represents the difference in labelling after preincubation with TEPP (B) and with TEPP plus Mipafox (C) (see Table 1).

Concn. of TEPP (μM)	Concn. of Mipafox (μM)	Difference (B) – (C) ($\mu\mu$moles of ^{32}P/g. of brain)
6·4	200	47
12·8	200	44
25·6	200	35
204·0	200	29
16	16	23
16	32	31
16	64	38
16	128	43
16	256	48

Table 3. *Comparison of the effects of Mipafox and Butafox during preincubation on subsequent phosphorylation of hen brain homogenates by* [^{32}P]*DFP*

Experimental conditions for the standard test were as described in the Materials and Methods section, with brain of a normal hen. Fixed inhibitor concentrations were TEPP (16 μM for preincubation) and [^{32}P]DFP (32 μM). Mipafox or Butafox in the range of concentrations quoted was present during preincubation. With TEPP as the only preincubation inhibitor, labelling was 176 $\mu\mu$moles/g. of brain (B). Tabulated results are the difference (B) – (C) between this quantity and that found when the second inhibitor was included in the preincubation.

Concn. of Mipafox (μM)	Difference in labelling (B) – (C) ($\mu\mu$moles/g.)	Concn. of Butafox (μM)	Difference in labelling (B) – (C) ($\mu\mu$moles/g.)
64	38	0·2	41
128*	43	0·8	43
256	48	3·2	52

*Concentration in the standard test.

Table 4. *Effects of a range of Butafox concentrations during preincubation on the subsequent phosphorylation by [^{32}P]DFP of brain homogenates from hens dosed with organophosphorus compounds*

Experimental conditions were as described for the standard test in the Materials and Methods section, with TEPP (16 μM for preincubation) and DFP (32 μM) but a range of Butafox concentrations substituted for Mipafox. Hens were dosed and killed as indicated in Table 1. Control results are the means of two values agreeing within 2%. MOCP, 2-Methylphenyl diphenyl phosphate.

Concn. of Butafox *in vitro* (μM)	Compound administered *in vivo* ... Labelling after preincubation with TEPP only ($\mu\mu$moles/g.)	Decrease in labelling due to addition of Butafox (B) – (C) ($\mu\mu$moles/g.) None	Paraoxon (non-neurotoxic)	MOCP (neurotoxic)	Mipafox (neurotoxic)
		176	127	91	115
0·2		41	—	—	3
0·8		43	35	1	4
3·2		52	37	5	2
12·8		68	43	6	6
51·2		85	50	18	19

effect, but that this decrease is far less by the time the lesion appears. The restoration appears to be not unlike that for all the other phosphorylation sites in brain.

Validity of the conditions of the standard test. The success of the standard test in classifying compounds so far suggests that it is valid. Nevertheless it was important to show that the association of neurotoxicity with a quantity of phosphorylation sites was not achieved by using insufficient quantities of TEPP or excessive quantities of Mipafox. Table 2 (top) shows that a 32-fold change of TEPP concentration around the usual 16 μM did not greatly decrease the value of (B) – (C), and that when Mipafox concentrations down to one-eighth of the standard 128 μM were used at least half the sites measured by (B) – (C) were still detected.

Effect of substituting Butafox for Mipafox in the standard test. Table 3 shows that concentrations of Butafox 160–320 times lower than those of Mipafox has a similar effect on (B) – (C). This correlates well with the ratio of the reported neurotoxic doses for these two compounds (0·10 and 25 mg./kg. respectively; ratio 250; Davies, Holland & Rumens, 1966) and strengthens the association between the value of (B) – (C) and neurotoxicity.

Table 4 shows that (B) – (C) measured for normal brain increased continuously as the Butafox concentration was increased from 0·8 μM (equivalent to 128 μM-Mipafox as used in the standard test) to 51·2 μM. The extra sites measured by concentrations of Butafox up to 12·8 μM were not very sensitive to a dose of Paraoxon *in vivo*. Also, with homogenates from birds dosed with two neurotoxic compounds very few sites were detected by Butafox at concentrations up to 12·8 μM. It is likely therefore that the 68 $\mu\mu$moles/g. detected in a normal homogenate by 12·8 μM-Butafox is a better measure of the total quantity of neurotoxic sites than the value of 43 $\mu\mu$moles/g. measured by 0·8 μM-Butafox or 128 μM-Mipafox.

Chemical nature of the phosphorylation site

The phosphorylation site that was indicated by the preceding experiments could possibly be on macromolecular lipid, protein, nucleic acid or carbohydrate. The acids or organic solvents used to inactivate reactions in the homogenate and to remove excess of DFP remove most low-molecular-weight cell components and (with organic solvents) a considerable amount of lipids. A value of 32 $\mu\mu$moles/g. obtained in the standard test on one normal brain homogenate was not significantly decreased when the washed precipitate was further washed once with chloroform–methanol–ether (2:1:1, by vol.) to increase lipid extraction and extracted with 0·45 M-perchloric acid at 90° for 15 min. to remove nucleic acids. The possibility that the phosphorylation site is on a carbohydrate has not been excluded. However, since low concentrations of DFP phosphorylate some esterases (see the introduction), it seems likely that the site is on a protein. Direct proof that this is so must await purification of the material.

Significance of quantity of 'neurotoxic site'

Since it has been suggested that the specific phosphorylation site may have esteratic activity it is of interest to consider whether the calculated content of neurotoxic site in normal brain is at all similar to the brain content of known esterases. A rough estimate of the content of acetylcholinesterase and butyrylcholinesterase can be obtained. According to Davison (1953) the I_{50} values for inhibition of acetylcholinesterase and butyryl-

cholinesterase of hen brain by DFP are 0·16 and 0·002 μM respectively at pH 7·8 and 37° for 30 min. reaction. Under those conditions both enzymes would be virtually 100% inhibited by 0·7 μM-DFP. A similar result would be expected with 2 μM-DFP at pH 8·0 and 25°, assuming negligible effect of the small pH change and a temperature coefficient for the first-order inhibition reaction of about 2·5/10°. It was found that both hen brain cholinesterases are fully inhibited after 30 min. incubation with eserine (1 μM) at 25° and pH 8·0, determined manometrically as described by Aldridge (1954), and no enzymes other than the cholinesterases are known to be inhibited by eserine under these conditions. It follows therefore that if comparison is made of the binding of DFP to brain preincubated with and without 1 μM-eserine then a difference should be observed that should be maximal at DFP concentrations of 2 μM or above, which will represent the quantity of the active cholinesterases originally present. Table 5 shows that a maximum is reached when the [^{32}P]DFP concentration is about 0·4–6 μM; this is in accord with the concentration calculated above. The value of 136–156 $\mu\mu$moles of binding site/g. can be taken to represent the brain content of cholinesterases, and it is encouraging that this is of the same order as the 68 $\mu\mu$moles of site/g. that has been found to represent the total quantity of unknown 'neurotoxic enzyme' (Table 4).

Table 5. *Effects of preincubation with eserine on labelling of hen brain homogenates by* [^{32}P]*DFP*

Experimental conditions were as described in the Materials and Methods section, except that incubation was continued for 30 min. after the addition of [^{32}P]DFP. The concentration of eserine was 1 μM.

Concn. of DFP (μM)	^{32}P bound ($\mu\mu$moles/g. of brain) after incubation with: (A) Buffer	(B) Eserine	Difference (A)−(B)
0·025	110	69	41
0·10	167	94	73
0·40	293	157	136
1·6	455	301	156
6·3	700	563	137
25	1190	1070	120

Standard test applied to various nervous tissues

Table 6 shows that the activity (B) − (C) can be found in hen brain and ox brain stored for a week at −20°. It is also present in fresh hen spinal cord, which is the site of the clinical lesion. The amount in spinal cord represents about 4·5% of the total phosphorylation sites found when inhibitors are omitted from the preincubation compared with 5·5% for hen brain and 4% for stored ox brain. In other experiments hen serum was phosphorylated by DFP but there was no difference between (B) and (C). Other non-nervous tissues have not been examined.

Studies in young hens

It has previously been shown that ataxia cannot be produced by single doses of neurotoxic organophosphorus compounds given to hens less than 80 days old (J. M. Barnes, personal communication; Barnes & Denz, 1953; Bondy *et al.* 1960). It was therefore surprising to find the specific binding sites in brains from young (40-day-old) hens and

Table 6. Phosphorylation of various nervous tissues by [^{32}P]*DFP under the conditions of the standard test*

Tissues and extracts were prepared and the standard test was performed as described in the Materials and Methods section. All values are means of duplicates agreeing within 2%.

Tissue	^{32}P bound ($\mu\mu$moles/g. wet wt. of original fresh tissue) after preincubation with: (A) Buffer only	(B) TEPP	(C) TEPP+Mipafox	Standard test difference (B)−(C) ($\mu\mu$moles/g.)
Normal adult hen brain (mean ± S.D. for eight hens)	668 ± 149	215 ± 103	178 ± 102	37 ± 8
Whole hen brain stored at −20° for 7 days	468	145	114	31
Ox brain stored −20° for 14 days				
Cortex	362	134	120	14
White matter	323	106	93	13
Hen spinal cord	283	108	95	13
Brain from normal 40-day-old chicks	659	169	130	39
	612	155	122	33
Brain from 40-day-old chicks 24 hr. after one dose of 2-methylphenyl diphenyl phosphate (600 mg./kg. orally)	380	91	86	5
	337	84	79	5

Table 7. *Phosphorylation of centrifuged brain extracts by [^{32}P]DFP under the conditions of the standard test*

Extracts were prepared and the standard test was performed as described in the Materials and Methods section. Centrifugations were at 105000*g* for 60min. All values are means of duplicates agreeing within 2%.

Tissue	^{32}P bound ($\mu\mu$moles/g. of original fresh tissue) after preincubation with: (A) Buffer only	(B) TEPP	(C) TEPP+Mipafox	Standard test difference (B)−(C) ($\mu\mu$moles/g.)
Supernatant from homogenate in 0·3M-sucrose	—	45	40	5
Sedimented particles from same homogenate	—	310	275	35
Supernatant from deoxycholate extract	514	176	162	14
Supernatant from ultrasonic extract of freeze-dried brain	—	130	113	17
	—	133	116	17

that these were eliminated by a single oral dose of 2-methylphenyl diphenyl phosphate (Table 6). It is therefore of particular importance to the standing of the current hypothesis that it has now been shown that four to seven doses of this compound (250mg./kg. orally) or six doses of Butafox (120μg./kg. subcutaneously) given during a period of 9 days to 40-day-old hens produces severe ataxia within 14 days of commencement of dosing (M. K. Johnson, unpublished work,). This regime prolongs the period during which the phosphorylation site is inhibited, although it is not clear why this should be necessary for production of ataxia.

Solubilization of phosphorylation sites associated with neurotoxicity

Table 7 shows that most phosphorylation sites, including those associated with neurotoxicity, are sedimented by centrifugation of a sucrose homogenate at 105000*g* for 60min. However, considerable activity was obtained in supernatants obtained by similar centrifugation of ultrasonic extracts of freeze-dried whole brain or of homogenates treated with sodium deoxycholate. It is not certain whether the activity is in true solution, but preliminary experiments have shown that some of the labelled proteins in these extracts can be separated by chromatography on ion-exchange cellulose.

GENERAL DISCUSSION

As a result of the standard test developed in this work the neurotoxic effect of organophosphorus compounds has been correlated with covalent binding of the inhibitor to a macromolecular site in hen brain. This site is probably a protein and has been shown to be present in spinal cord, where the clinical lesion occurs. The fact that the site reacts with low concentrations of some but not all organophosphorus compounds supports the view that it may be a specific enzyme. Moreover, the concentration of this site is similar to that of two other brain esterases (the cholinesterases).

It would be useful if the labelling technique could be further improved so that the only sites labelled were on the neurotoxic 'enzyme'. Preliminary experiments with radioactive Butafox instead of DFP have shown that over 60% of the labelling achieved after preincubation with a mixture of non-neurotoxic inhibitors is associated with the neurotoxic 'enzyme'. Some irrelevant sites may also be removed by including ester substrates in the preincubation.

The problem of neurotoxicity of organophosphorus compounds has been tackled with a few inhibitors only. Others have been examined and discarded for a variety of reasons, but the technique of successive elimination of active sites by stepwise addition of irreversible inhibitors is very versatile, and can be used to study any enzyme in any crude mixture provided that inhibitors can be devised. The final addition can be a radioactively labelled irreversible inhibitor or a potential substrate. Enzymes are usually discovered by the observation of substrate conversion, and several workers have shown how useful irreversible inhibitors are to identify several enzymes in a tissue when each has overlapping substrate specificity (Aldridge, 1953*a*,*b*, 1954; Myers, 1956). The present technique is a logical extension in that no prior knowledge of substrate specificity is required, yet an enzyme may be quite clearly identified and distinguished from others whose substrate requirements may or may not be known. Admittedly this is only a means of bringing study material to light. Identification of an enzyme merely by its behaviour towards inhibitors is a long way from understanding its catalytic capacity or physiological function. It does, however, provide an alternative approach to these problems and to the separation of enzymes, and Ramachandran, Engstrom & Agren (1963) have separated numerous liver esterases after unselective labelling by [^{32}P]DFP.

I thank Miss H. Mackenzie for skilled technical assistance, Mrs J. Jenkins for help in neurotoxicity testing and Dr W. N. Aldridge for much fruitful discussion.

REFERENCES

Aldridge, W. N. (1953*a*). *Biochem. J.* **53**, 110.
Aldridge, W. N. (1953*b*). *Biochem. J.* **53**, 117.
Aldridge, W. N. (1954). *Biochem. J.* **57**, 692.
Aldridge, W. N. (1956). *Rep. Progr. Chem.* **53**, 294.
Aldridge, W. N. & Barnes, J. M. (1961). *Biochem. Pharmacol.* **6**, 177.
Aldridge, W. N. & Barnes, J. M. (1966). *Biochem. Pharmacol.* **15**, 549.
Aldridge, W. N. & Barnes, J. M. (1967). *Proc. Europ. Soc. Study of Drug Toxicity*, vol. 8, p. 162; *Excerpta med. int. Congr. Series no.* 118.
Aldridge, W. N., Barnes, J. M. & Johnson, M. K. (1969). *Proc. Soc. N.Y. Acad. Sci.* (in the Press).
Aldridge, W. N., Emery, R. C. & Street, B. W. (1960). *Biochem. J.* **77**, 326.
Barnes, J. M. & Denz, F. A. (1953). *J. Path. Bact.* **65**, 597.
Bondy, H. F., Field, E. J., Worden, A. N. & Hughes, J. P. W. (1960). *Brit. J. industr. Med.* **17**, 190.
Bruno, G. A. & Christian, J. E. (1961). *Analyt. Chem.* **33**, 1216.
Cavanagh, J. B. (1964). *Int. Rev. exp. Path.* **3**, 219.
Davies, D. R., Holland, P. & Rumens, M. J. (1960). *Brit. J. Pharmacol.* **15**, 271.
Davies, D. R., Holland, P. & Rumens, M. J. (1966). *Biochem. Pharmacol.* **15**, 1783.
Davison, A. N. (1953). *Brit. J. Pharmacol.* **8**, 212.
Johnson, M. K. (1967). *Abstr. 1st. int. Meet. int. Soc. Neurochem., Strasbourg*, p. 111.
Myers, D. K. (1956). *Biochem. J.* **64**, 740.
Ramachandran, B. V., Engstrom, L. & Agren, G. (1963). *Biochem. Pharmacol.* **12**, 167.